TANK2, a new TRF1-associated PARP, causes rapid induction of cell death upon overexpression

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Tankyrase (TANK 1) is a human telomere-associated poly (ADP-ribose) polymerase (PARP) that binds the telomere binding protein TRF1 and increases telomere length when overexpressed. Here we report characterization of a second human tankyrase, tankyrase 2 (TANK2), which can also interact with TRF1 but has properties distinct from those of TANK1. TANK2 is encoded by a 66 kb gene (*TNKS2*) containing 28 exons, which express a 6.7 kb mRNA and a 1166 amino acid protein. The protein shares 85% amino acid identity with TANK1 in the ankyrin repeat, sterile alpha motif and PARP catalytic domains, but has a unique N-terminal domain, which is conserved in the murine *TNKS2* gene. TANK2 interacted with TRF1 in yeast and *in vitro* and localized predominantly to a perinuclear region, similar to the properties of TANK1. In contrast to TANK1, however, TANK2 caused rapid cell death when highly overexpressed. TANK2-induced death featured loss of mitochondrial membrane potential, but not PARP1 cleavage, suggesting that TANK2 kills cells by necrosis, and was prevented by the PARP inhibitor 3-aminobenzamide. In vivo, TANK2 may differ from TANK1 in its intrinsic or regulated PARP activity or its substrate specificity.

[1] **The abbreviations used are:** 3AB, 3-aminobenzamide; β-gal, β-galactosidase; BAC, bacterial artificial chromosome; DAPI, 4', 6-diamidino-2-phenyl-indole; FISH, fluorescence in situ hybridization; NTD, N-terminal domain; ORF, open reading frame; PARP, poly ADP-ribose polymerase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RLM, RNA ligase-mediated; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TANK, telomere-associated, ankyrin-repeat-containing PARP (tankyrase; non-classical PARP); TANK1, tankyrase 1; TANK2, tankyrase 2; UTR, untranslated region.

INTRODUCTION

Telomeres are the repetitive DNA sequences and specialized proteins that cap the ends of linear chromosomes and protect them from end-to-end fusion. In mammalian cells, loss or disruption of a telomere can cause cellular senescence, cell death or genomic instability, depending on the genotype and cell context. A variety of events can lead to dysfunctional telomeres. Telomeres can be damaged directly by genotoxic agents and/or faulty DNA repair processes. In addition, the telomeric structure can be disrupted by changes in the expression or function of certain telomere-associated proteins. Finally, telomeres can erode owing to the biochemistry of DNA replication, which leaves 50-200 bp of 3' telomeric DNA unreplicated upon completion of each S phase. Thus, in the absence of the enzyme telomerase, or another mechanism to replenish telomeric DNA, proliferating cells progressively lose telomeric DNA and eventually acquire one or more critically short or dysfunctional telomeres (1-3).

Most normal mammalian cells respond to a critically short or dysfunctional telomere by undergoing cellular senescence (4-7). This process results in an irreversible arrest of cell proliferation and striking changes in cell function (8). Dysfunctional telomeres can also induce apoptotic cell death, particularly in cells that harbor mutations in one or more cell cycle or DNA damage checkpoint (9-11). Very little is known about how telomeres signal cells to undergo senescence or apoptosis. However, the recent discovery of a telomere-associated poly (ADP-ribose) polymerase (PARP)[1] (12) provides a potential mechanism by which telomeres transmit signals to cellular proteins that regulate the senescence and apoptotic responses.

PARPs catalyze the formation of branched chains of ADP-ribose polymers on selected proteins, using NAD+ as a substrate (13,14). Classic PARPs (PARPs 1-3) are activated in

response to single- or double-strand DNA breaks, whereupon they ADP-ribosylate a number of proteins, including key regulators of transcription, cell cycle progression and DNA repair. The ADP-ribosylation is transient and can either stimulate or inhibit the activity of the target proteins. PARP activation provides a rapid, post-translational signal that can halt the transcription and replication machineries and mobilize DNA repair machineries. PARPs are also important for suppressing recombination at DNA ends (15,16), and participate in anchoring chromatin to the nuclear matrix (17-19), where certain DNA repair and recombination processes appear to occur (20,21).

Because telomeres are DNA ends that are anchored to the nuclear matrix (19,22), and appear to elicit a DNA damage response when dysfunctional, PARPs are also thought to participate in telomere maintenance and/or transmitting signals generated by dysfunctional telomeres. Consistent with this view, cells from knockout mice that lack PARP1, a classic PARP encoded by the *ADPRT1* gene, have somewhat shorter (30%) telomeres than wild-type cells (15). Interestingly, cell lysates from the knockout mice have residual PARP activity (23). This finding, and the mild telomere phenotype of *ADPRT1* -/- mice, suggested that one or more PARP distinct from PARP1 may more directly participate in telomere maintenance and/or signaling. Thus far, the strongest candidate for such a PARP is tankyrase (12), referred to here as TANK1.

TANK1 (encoded by the *TNKS* gene on human chromosome 8) (12,24) is a non-classic PARP that interacts with and ADP-ribosylates the telomere-binding protein TRF1 (12). TANK1 lacks a nuclear localization signal, and shows a predominantly perinuclear and cytoplasmic distribution, although it is found at the telomeres of metaphase chromosomes in cells that overexpress TRF1. *In vitro*, ribosylation by TANK1 displaces TRF1 from telomeric DNA (12).

This finding, and the phenotype of cells that express a dominant negative TRF1 mutant (25), suggested that TANK1 might be a positive regulator of telomere length in telomerase-expressing cells. Indeed, when overexpressed, a nuclear-targeted TANK1 protein increased telomere length in telomerase-positive tumor cells (26).

Here, we describe the characterization of a second tankyrase-like protein, TANK2, recently identified as a Golgi-associated protein (also referred to as TNKL) (27,28). TANK2 is encoded by a distinct gene (*TNKS2*) on human chromosome 10. It shares >80% overall amino acid identity, and a similar intracellular distribution pattern, with TANK1. However, TANK2 contains a unique N-terminal domain, which is also conserved in the murine *TNKS2* gene. In contrast to TANK1, however, TANK2, when overexpressed, induced rapid cell death with features of necrosis. Although there is as yet no direct evidence that TANK2 has PARP activity, we further show that TANK2-induced cell death is prevented by the general PARP inhibitor 3-aminobenzamide. Our results raise the possibility that TANK 2 may be a more active PARP than TANK1 and/or have unique substrate specificities, either attribute of which enables it to signal cell death.

EXPERIMENTAL PROCEDURES

Cells and cell culture -- WI-38 and HT1080 cells were obtained as described (29). 82-6 normal human fibroblasts were from Dr. J. Oshima (U. Washington), and VA13 cells were from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagles medium and 10% fetal calf serum, as described (29).

Yeast two-hybrid screening -- A partial *TNKS2* cDNA was cloned from a human fibroblast yeast two-hybrid library using TRF1 as a bait. The library, vectors and screening methods have been described (30). Briefly, we transfected the library into yeast expressing the pGBT9-TRF1 bait vector, and screened 10^6 transformants on selective media containing 3-aminotriazole. Surviving colonies were tested for expression of the *LacZ* (β-galactosidase; β-gal) reporter. One colony (pGAD-TANK2-1.7) that survived selection and expressed β-gal contained a 1.7 kb insert, which we subcloned into pGEM-TA and sequenced. The insert was homologous to a central region of the *TNKS* cDNA. We named the gene encoding the insert *TNKS2*, and the corresponding protein TANK2. We mapped the TRF1 domain with which the protein fragment encoded by pGAD-TANK2-1.7 interacted using yeast two-hybrid analysis. Briefly, *TERF1* cDNA fragments cloned into pGBT9 (30) were introduced into yeast expressing pGAD-TANK2-1.7, and the level of β-gal reporter activity was quantified as described (30).

Cloning the complete TNKS2 open reading frame -- We generated a clone containing the *TNKS2* open reading frame (ORF) by 5' RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE) and 3' RACE. To obtain the 3' end, we used 3' RACE to screen a human placenta Marathon cDNA library (Clontech), and isolated a 2.7-kb fragment that we subcloned into pGEM-TA and sequenced. This fragment had 1 kb of sequence overlap with the pGAD-TANK2-1.7 insert, an additional 1.5 kb of coding sequence, a stop codon and 200 bp of the 3' untranslated region (UTR). To obtain the 5' end, we used a 5' RLM RACE kit (Ambion) to generate random primed cDNA libraries from human placenta (Clontech) and the human cell lines HT1080 and VA13. We obtained identical 450-bp products from all three libraries, determined by sequencing. The combined cDNA fragments generated a continuous 3.4 kb ORF, with approximately 200 bp each of 5' and 3' UTR (GenBank accession # AF342982). The

fragments were assembled into a single cDNA using the polymerase chain reaction (PCR) and restriction enzyme digestion and ligation. The assembled cDNA was cloned into pBluescript2SK+ (Stratagene), and then subcloned into vectors for epitope tagging or expression. The TANK2 ORF identified by RACE was identical to the ORF contained in three lambda cDNA clones, isolated from a human 293 tumor cell λgt11 library by screening with a 347 bp probe corresponding to the *TNKS2* ankyrin repeat region. The lambda clones encompassed 6.1 kb of the *TNKS2* cDNA. The partial (450 bp) mouse *TNKS2* cDNA was isolated from mouse testis polyA+ RNA (Ambion) using 5' RLM RACE and oligonucleotides corresponding to mouse ESTs with strong homology to human *TNKS2*. To determine the genomic organization of human *TNKS2*, we screened a human bacterial artificial chromosome (BAC) library (Research Genetics) with a SacII cDNA fragment containing the initiation ATG and 5' UTR of *TNKS2*. Four clones (534O13, 174E12, 126H3; 530L19) were determined to contain the entire gene by PCR amplification of sequences at the 5' and 3' ends of the cDNA. We also identified a BAC sequence encompassing *TNKS2* in the High Throughput Genomic Sequence database (Chromosome 10; GenBank accession # AL359707) which had sequence gaps. We filled the gaps by sequencing the appropriate PCR fragments of the BAC clones.

Chromosomal localization -- The pGAD-TANK2-1.7 insert was used to localize the *TNKS2* gene by fluorescence in situ hybridization (FISH) to DAPI (4', 6-diamidino-2-phenyl-indole)-banded metaphase chromosomes, as described (31). The *TNKS2* location was confirmed and refined by radiation hybrid mapping, as described (32). Briefly, we screened the medium resolution Stanford G3 panel using the primers GAT ACA CTC ACC GGA GAA AAG-3', and GTG AAC TGG ACA CCC AGT ACC-3', which amplified a 3 kb fragment from 13 out of 83

hybrid clones. These results were submitted to the Stanford RH server, which provided a map location and identified the closest marker.

Northern analysis -- Poly-A RNA (10 μg) was isolated (Qiagen) from HT1080 cells, separated, blotted onto a nylon membrane, and hybridized to *TNKS*- or *TNKS2*-specific probes, as described (33). Probes were generated by PCR using primers to amplify sequences encoding the unique TANK1 and TANK2 N-termini. The primers were CTC CCA ACC AGC CGG CAGT-3' and CCA GCA GTT CCC GTA GGG CCC-3' for TANK1, and ATG GGA CTG CGC CGG ATC CGG TGA CAG CAGG-3' and GTG GAG CCG GCC GCC CGA GA-3' for TANK2. A multiple tissue northern blot containing 2 μg poly-A RNA from selected human tissues was purchased from Clontech, hybridized with a 1 kb probe corresponding to the TANK2 ankyrin domain, and rehybridized with a β-actin probe, as described (30).

In vitro transcription and translation -- The *TNKS2* cDNA in pBluescript2SK± (0.2 μg DNA), *TNKS* cDNA in pBK-CMV (provided by T. deLange) (1 μg) and hTERT cDNA in pGRN125 (1 μg) were added to a coupled *in vitro* transcription/translation (IVT) reaction containing ^{35}S-methionine and rabbit reticulocyte lysate (Promega), as described (30). Reactions were run for 60 min at 30^{o} C. The translation products were separated by 6.5% SDS-PAGE and visualized by autoradiography.

Immunoprecipitation -- cDNAs encoding TANK1, TANK2 or epitope-tagged TRF1 (HA-TRF1) were transcribed and translated *in vitro* as described above, and co-immunoprecipitated as described (30). Briefly, *in vitro* translated TANK1 or TANK2 was mixed with an equal amount of *in vitro* translated HA-TRF1 in 0.5 ml binding buffer (20 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% Tween-20, Sigma

mammalian protease inhibitors) and incubated for 1 h at 4° C. Reactions were precleared with 25 µl protein A/G beads (Pierce), and the supernatant incubated with 5 µg anti-HA antibody for 4 h at 4° C. Protein A/G beads (25 µl) were added, and, after 1 h incubation at 4° C, the beads were collected by centrifugation and washed with binding buffer. Bound proteins were released by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and separated on 4-15% SDS-PAGE gels. ^{35}S signals were detected using a phosphorimager.

Western analysis -- Western analysis was carried out as previously described (34), using enhanced chemiluminescence (Amersham) and autoradiography. Mouse monoclonal antibodies were used to detect V5 (R96025; Invitrogen), FLAG (M2 or M5; Sigma), and PARP (33-3100; Zymed). Mouse monoclonal (575400; Calbiochem) or rabbit polyclonal (described below) antibodies detected both TANK1 and TANK2, as described in the text.

Antibody production -- We conjugated a peptide corresponding to amino acids 660-680 of TANK2 to keyhole limpet hemocyanin, and used the conjugate to produce polyclonal anti-serum in rabbits using a commercial service (Covance). The peptide was also used to generate an affinity column (Sulfolink; Pierce). Rabbit serum was applied to the column, and washed with 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl. Bound antibodies (Ab-591) were eluted with 100 mM glycine (pH 2.8), neutralized with 1 M Tris (pH 8), dialyzed against 10 mM Tris-HCl (pH 7.5), and stored at –80^{o} C.

Immunocytochemistry -- We immunostained cells as previously described (30,35). Briefly, cells grown on coverslips were fixed in 10% formalin, blocked in phosphate buffered saline containing 1% bovine serum albumin, and stained with the mouse anti-V5 or anti-FLAG

antibodies for 2 h at room temperature. Coverslips were washed and stained with fluorescein-conjugated goat-anti-mouse antibody and mounted in Vectashield containing DAPI (Vector Labs). Images were obtained using a cooled charged-couple device camera connected to an epifluorescence microscope.

Transfections and cell viability assays -- Cells on coverslips were transfected with the *TNKS* or *TNKS2* cDNAs (in pBK-CMV or pcDNA3.1, respectively), or the CMV-βgal normalization vector, using FuGene 6 (Roche), as instructed by the supplier. Transfection efficiency was estimated from CMV-βgal-transfected cultures by the fraction of β-gal positive cells, as described (33). Cell death was assessed by observing the number of adherent cells 6-10 h after transfection, and by staining with MitoCapture (Biovision), a fluorescent lipophilic cationic reagent that assesses mitochondrial membrane permeability, according to the supplier's instructions. Briefly, cells were incubated with the MitoCapture reagent for 15 min at 37^{o} C, and counted by fluorescence microscopy using a wide band-pass filter. Cells with intact mitochondria exhibited focal red cytosolic fluorescence, whereas cells with permeabilized mitochondria exhibited diffuse green cytosolic fluorescence. Cells lacking red fluorescence and having green fluorescence were scored positive.

RESULTS

Identification of TANK 2 -- We identified TANK2 in a yeast two hybrid screen (36) of a human fibroblast cDNA library using TRF1 as a bait (30). The screen yielded several positive colonies, one of which harbored a vector (pGAD-TANK2-1.7) containing a 1.7 kb insert with striking sequence homology to *TNKS*, a gene encoding the TRF1-interacting protein named

tankyrase or TANK1 (12,27). The 1.7 kb insert spanned the region corresponding to the TANK1 ankyrin repeat domain. Sequencing showed that it shared 81% nucleotide identity to *TNKS*, and 85% predicted amino acid identity to the TANK1 protein. This degree of homology, while highly significant, was sufficiently different to suggest that the 1.7 kb cDNA derived from a distinct, albeit related, gene. We refer to this gene as *TNKS2* and the protein encoded by it as TANK2. This gene was recently independently identified (and named *TNKL*) as encoding a protein (TANK2) that, together with TANK1, associates with the Golgi apparatus (27).

To isolate the entire *TNKS2* coding region, we used 5' RLM-RACE and 3'-RACE. We assembled a 3.8 kb cDNA containing a 3.4 kb ORF with putative translational start and stop codons, and approximately 200 bp each of the 5' and 3' untranslated regions (UTRs). The ORF encoded an 1166 amino acid protein that had 85% identity to the ankyrin repeats, sterile alpha motif (SAM) and PARP catalytic domain of TANK1 (Fig. 1A). However, TANK2 contained a unique 25 amino acid N-terminal domain (NTD) that replaced the larger histidine-proline-serine (HPS) rich domain in TANK 1 (Fig. 1A). To verify the position of the initiation codon, we isolated a partial mouse *TNKS2* (m*TNKS2*) cDNA encompassing the NTD and part of the 5' UTR. The NTD was nearly identical between the human and mouse TANK2 proteins, with only 2 conservative amino acid changes in the first 56 residues (96% identity; Fig. 1B). Both the murine and human *TNKS2* cDNAs have an ORF that extends downstream of the putative initiation ATG. They share 95% nucleotide identity throughout the 168 residues that encoded the first 56 amino acids, with 6 of the 8 mismatches occurring in the third position of codons. Upstream of the presumed initiation codon, the nucleotide identity declined abruptly (<83%) and the sequence mismatches were randomly distributed among all three codon positions (Fig. 1B).

This pattern of conservation strongly suggests that our human *TNKS2* cDNA contained the translational start site.

Chromosomal localization and genomic organization -- To confirm that TANK2 is not encoded by *TNKS*, we used FISH to map *TNKS2* to human chromosome 10q23-24 (not shown). This location was verified by radiation hybrid mapping, which localized *TNKS2* more precisely to 10q23.3, and identified the closest marker as D10S536 (Fig, 2A). This position agrees with a report that localized the gene (*TNKL*) identical to *TNKS2* near D10S2170 (37). Since TNKS is located on chromosome 8 (24), these findings verify that *TNKS2* is distinct from *TNKS*.

To determine the genomic organization of *TNKS2*, we analyzed the High Throughput Genomic Sequence database and identified *TNKS2* sequences encompassing most of the gene, albeit with some sequence gaps. To close the gaps, we screened a human BAC library, obtained four BACs containing the *TNKS2* gene, and sequenced the relevant segments. The *TNKS2* gene is approximately 66 kb in size, organized into 26 introns and 27 exons, all of which contain coding sequences and two of which also contain 5' and 3' UTR sequences (Fig. 2B). The exons range from 37 to 484 bp, whereas the introns vary greatly in size, from 89 bp to 14 kb.

TNKS2 mRNA expression -- To determine the expression pattern of *TNKS2* among human tissues, we probed a northern blot containing poly-(A) RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas with a cDNA probe corresponding to a 1 kb region encompassing the ankyrin domain of TANK2. The probe detected a single 6.6 kb mRNA species (Fig. 3A). *TNKS2* mRNA was expressed in all the tissues tested (including liver, evident on longer exposures), albeit to varying levels. In addition, a different multi-tissue northern blot

showed that the mRNA was expressed in spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes (not shown). Thus, the *TNKS2* appeared to be ubiquitously expressed among human tissues.

Because the TANK2 probe used in this analysis shared significant sequence similarity to TANK 1, we designed cDNA probes corresponding to the unique N-terminal domains of TANK1 and TANK2. We hybridized these probes to northern blots of polyadenylated RNA from cultured HT1080 fibrosarcoma cells. As expected (12), the TANK1-specific probe hybridized to multiple mRNA species, ranging in size from 2 to 9 kb (Fig. 3B). By contrast, the TANK2-specific probe hybridized to a single 6.6 kb mRNA species (Fig. 5B). This size is consistent with the analysis of human tissue RNA (Fig. 3A), and with the size of the *TNKS2* cDNA contained in the lambda clones. These results verify that TANK1 and TANK2 are produced from different mRNAs, and indicate that *TNKS2*, in contrast to *TNKS*, generates only a single major mRNA species.

TANK2 protein expression -- To verify that the predicted ORF in the *TNKS2* cDNA is correct and characterize the TANK2 protein, we transcribed and translated the *TNKS* and *TNKS2* cDNAs *in vitro*, and analyzed the radiolabeled translation products by SDS-PAGE (Fig. 4A). Both cDNAs produced a single labeled protein. As expected (12), TANK1 migrated with an apparent molecular weight of 150-160 kD. TANK2 migrated somewhat faster, with an apparent molecular weight of 130-135 kD, consistent with the 127 kD size predicted from the ORF.

To analyze endogenous TANK proteins, we raised a rabbit polyclonal antiserum against a peptide corresponding to amino acids 660-680, a conserved sequence in the ankyrin domains of

TANK1 and TANK2. This antiserum (Ab-591) was affinity-purified, and used to analyze western blots of cell lysates from normal human fibroblasts (WI-38) and the human tumor cell lines HT1080 and VA13 (Fig. 4B). In all cells, the antibody detected two major proteins, which generated signals of nearly equal intensity. These proteins corresponded in size to the *in vitro* translation products (Fig. 4A), and to those predicted from the TANK1 and TANK2 ORFs. Similar results were obtained with a commercially available antibody (not shown), except the TANK1 signal was about 5-fold more intense than the TANK2 signal. To confirm the identities of the proteins detected by the antibodies, we transiently transfected TANK1 and TANK2 expression vectors into HT1080 cells, and analyzed cell lysates by western blotting (Fig. 4B). The TANK1 expression vector generated a prominent 150-160 kD band, whereas the TANK2 vector generated a prominent 130-135 kD band.

Interaction with TRF1 -- Our yeast two-hybrid screen indicated that TANK2, like TANK1, is a TRF1-interacting protein, although previous reports either did not test (27) or could not demonstrate (28) interaction with TRF1. To verify that TANK2 and TRF1 interact, we produced radiolabeled epitope (HA)-tagged TRF1 (30), untagged TANK1, and untagged or epitope (V5 or FLAG)-tagged TANK2 by *in vitro* transcription and translation (Fig. 5A). We then combined the HA-TRF1 with the TANK1 or TANK2 translation products, immunoprecipitated with anti-HA or control antibodies, and identified proteins in the immunoprecipitates by SDS-PAGE and autoradiography (Fig. 5B). As expected, anti-HA (Fig. 5B, lane 1), but not control antibody (not shown), precipitated TANK1. In addition, anti-HA, but not control antibody, also precipitated unmodified or FLAG-tagged TANK2 (Fig. 5B, lanes 3, 4 and 6). These results indicate that TANK2, like TANK1, interacts with TRF1. We were

unable to co-precipitate TRF1 and TANK2 from cell lysates. However, when cells were lysed in non-denaturing immunoprecipitation buffers, the majority of TANK2 was found in the insoluble material (not shown). This result suggests that TANK2 associates with the relatively insoluble nuclear or cytoskeleton matrices, and may explain why we could not precipitate it with TRF1 from non-denaturing cell lysates.

To identify the domain on TRF1 that interacts with TANK2, we used the yeast two hybrid system (36) to test TRF1 fragments (30) for their ability to interact with the TANK2 fragment encoded by pGAD-TANK2-1.7. TANK2 interacted with a 52 amino acid domain in the TRF1 N-terminus (Fig. 5C). This TRF1 domain was also shown to interact with TANK1 (12). These results suggest that TANK1 and TANK2 bind the same region in TRF1, and thus may compete for interaction with TRF1.

Subcellular localization -- Because Ab-591 and the commercial antibody did not distinguish TANK1 from TANK2, we transfected a V5 (C-terminal) epitope-tagged TANK2 expression vector into cells, and determined the subcellular localization of the V5 epitope by indirect immunofluorescence (Fig. 6). In both normal human fibroblasts (82-6, Fig. 6A and WI-38, not shown) and human fibrosarcoma cells (HT1080, Fig. 6B), TANK2 was detected as punctate perinuclear staining in the cytoplasm, and staining at the nuclear boundary. This distribution is similar to that reported for TANK 1 (12). It is consistent with localization to Golgi vesicles, as recently reported (27), and the nuclear membrane and pores, as reported for TANK1 (38). Interestingly, TANK2 was more diffusely perinuclear in normal cells, in contrast to a "capped" perinuclear distribution in fibrosarcoma cells (Fig. 6), suggesting possible differences between normal and tumor-derived cells. In mitotic cells, TANK2 localized to the

pericentriolar matrix, as observed for TANK1 (Fig. 6C). Figure 6B shows the staining pattern in HT1080 cells that overexpress TRF1 (30), but an identical staining pattern was seen in unmodified HT1080 cells (not shown). Thus, in contrast to TANK1, TANK2 remained largely perinuclear in TRF1-overexpressing cells.

Overexpression of TANK2 causes cell death -- When overexpressed, TANK1 gradually lengthens telomeres in telomerase-positive cells, presumably by inhibiting TRF1 function (26). To determine whether TANK2 affected telomeres, we attempted to stably overexpress it using recombinant retroviruses or DNA transfection. These attempts failed, or yielded clones with low expression, in both normal and tumor-derived cells. This result raised the possibility that TANK2 overexpression is lethal to cells.

To test this possibility, we transiently transfected control, TANK1 and TANK2 expression vectors into cells and monitored viability using the MitoCapture probe. This reagent produces red punctate (mitochondrial) fluorescence when the mitochondrial membrane potential is intact (negative with respect to the cytoplasm), and green diffuse cytoplasmic fluorescence when mitochondrial membrane permeability is compromised and the membrane potential collapses (39) (Fig. 7A). The TANK2, but not the TANK1 or control, vector caused a rapid loss of mitochondrial membrane potential within 7 h after transfection (Fig. 7A, B). At this time, TANK1 and TANK2 were both abundantly expressed (see Fig. 4B). At later times (24-36 h after transfection), virtually no TANK2-expressing cells were detected, as judged by immunostaining for the epitope tag (not shown). These results suggest that overexpression of TANK2, but not TANK1, causes rapid loss of mitochondrial membrane potential, followed by cell death.

We determined the efficiency of TANK2 cell killing by transfecting parallel cultures with a CMV-βgal vector, and staining for β−galactosidase. Transfection efficiencies were about 10% for normal fibroblasts and 30-40% for HT1080 fibrosarcoma cells (not shown). When normalized for transfection efficiency, overexpressed TANK2 collapsed the mitochondrial membrane potential in >60% of transfected cells within 7 h. At this time, western analysis showed that TANK1 and TANK2 were highly expressed (Fig. 4B). There was no difference between normal and malignant cells in their susceptibility to killing by overexpressed TANK2 (Fig. 7B). Moreover, TANK2 killed cells regardless of whether it was untagged (Fig. 7) or epitope-tagged at the N- (FLAG) or C- (V5) terminus (not shown).

To determine whether TANK2-induced cell death had features of apoptosis or necrosis, we assessed the integrity of PARP1, which is cleaved shortly after apoptotic cell death is initiated (40,41). PARP1 remained intact up to 24 h after HT1080 cells were transfected with the TANK2 expression vector (Fig. 7C), indicating that cell death probably occurs by necrosis.

PARP activity can deplete intracellular NAD+, and subsequently intracellular ATP, thereby causing necrotic cell death (42). Moreover, high PARP activity can cause cells undergoing apoptosis to switch to necrotic cell death, while PARP inhibition can cause cells undergoing necrosis to switch to apoptotic cell death (42,43). To obtain an indication as to whether TANK2-induced lethality depends on its putative PARP activity, we transiently expressed TANK2 in HT1080 cells in the presence 3-aminobenzimide (3AB), a general PARP inhibitor (44). 3AB substantially diminished TANK2-induced cell death, as indicated by the MitoCapture assay (Fig. 8A). Even 24 h after transfection, very few cells that received the TANK2 expression vector in the presence of 3AB had detached from the culture dish, in contrast

to those that did not receive 3AB. 3AB did not interfere with expression of the transfected vector because western analysis showed that TANK2 was expressed to the same extent in the absence or presence of 10 mM 3AB, and that 20 mM 3AB only slightly suppressed expression (not shown). Moreover, the ability of 3AB to protect cells from death was not complete or permanent. TANK2-expressing cells cultured in the presence of 10 mM 3AB eventually died, generally within a few days after transfection (Fig. 8B). These results indicate that inhibition of the putative PARP activity only partially protected cells from TANK2-induced death.

DISCUSSION

The classic PARPs (PARPs 1-3) play important roles in the cellular responses to DNA damage. Although many proteins stimulate PARP activity, it is not yet known how PARPs sense DNA damage. PARPs bind DNA, protect free DNA ends, and modify chromatin by ADP-ribosylating proteins such as histone H1 (14). Activated PARPs ADP-ribosylate many proteins, including key components or regulators of DNA replication, transcription and repair. PARP substrates include DNA polymerases, topoisomerases and ligases; HMG (high mobility group) proteins and transcription factor such as FOS; and p53, XRCC1, PCNA and the DNA-dependent protein kinase catalytic subunit (14,45). ADP-ribosylation can activate or inhibit protein function, depending on the substrate. Thus, PARPs transduce signals from damaged DNA to cellular machineries that regulate gene expression, cell cycle progression and DNA repair.

The TANKs and classic PARPs, share a number of differences and similarities regarding function. Like the classic PARPs, the non-classic PARPs TANK1 and TANK2 may transduce signals from dysfunctional telomeres, and thus play a role in regulating cellular senescence and

genomic stability. On the other hand, because TANKs localize predominantly outside the nucleus and interact with Golgi proteins (27,28), they may have distinct non-nuclear functions. Likewise, TANK1 and TANK2 share both differences and similarities.

First, PARPs have not been shown to interact directly with telomeres or telomere binding proteins, although PARP deficiency causes telomere shortening (15,46). By contrast, both TANK1 (12) and TANK2 (Fig. 5) interact with TRF1. Although a recent report found no interaction between the TANK2 ankyrin domain and TRF1 in a Gal4/LexA two-hybrid assay (28), we cloned TANK2 from a Gal4/Gal4 two-hybrid screen for TRF1-interacting proteins. We also used two-hybrid analyses to show that TANK2 interacts with the same TRF1 domain that binds TANK1. Moreover, *in vitro* translated TANK2 and TRF1 co-immunoprecipitated, indicating that these proteins can interact.

Second, PARPs are almost entirely nuclear, having a diffuse nucleoplasmic distribution (40). By contrast, both TANK1 (27,38) and TANK2 (Fig. 6) localize predominantly to the perinuclear cytoplasm and nuclear boundary, with only a small fraction evident in the nucleus. Both TANKs lack a known nuclear localization motif, and TANK1 is largely extra-nuclear unless TRF1 is overexpressed (38). TANK2, in contrast, did not localize to the nucleus in TRF1-overexpressing cells, despite its ability to bind TRF1. TRF1 may have a higher affinity for TANK1, relative to TANK2, or TANK2 may interact more strongly with perinuclear or nuclear membrane proteins than TRF1. Whatever the case, in the absence of overexpressed TRF1, both TANKs localize predominantly outside the nucleus, suggesting that only a small fraction can associate with telomeres. Immuno-labeling and electron microscopy showed that TANK1 associates with nuclear pores (26). TANK2 had not been identified when this study was

performed, and the antibodies might also have recognized TANK2. Until specific antibodies are developed, it is not clear whether TANK1, TANK2 or both localize to nuclear pores.

Recent data suggest that TANKs also localize to Golgi vesicles. Both TANKs interacted with the insulin responsive amino peptidase (IRAP), a component of Glut4 Golgi vesicles (27). In addition, TANK2 was identified as an interacting partner of GRB14, a Src homology 2 domain-adapter protein, and showed some, but not complete, co-localization with Golgi vesicles (28). We also observed TANK2 in perinuclear foci, consistent with localization to Golgi and possibly other cytoplasmic vesicles. The significance of the localization is not yet known. TANKs may participate in Golgi or endosome vesicle trafficking (28), or may bring proteins to the nuclear membrane for import.

Despite their dissimilar intracellular localization during interphase, both TANK1 and PARP1 were observed at centrosomes during mitosis (38,47). TANK2 also localized to mitotic centrosomes. Classic and non-classic PARPs may share a role in centrosome or spindle function, or a common storage site during mitosis. In addition, both PARP1 and TANK1 can, under at least some circumstances, influence telomere length. Germline inactivation of PARP1 in mice modestly reduced telomere length (15), whereas overexpression of nuclear targeted-TANK1 in human tumor cells modestly increased telomere length (26). Finally, both PARP1 and TANK2 cause necrotic cell death when overexpressed or, in the case of PARP1, activated by DNA damage (42). PARP1 is thought to kill cells by depleting intracellular ATP, a consequence of its utilization of NAD+ for ADP-ribosylation (42). TANK2 may cause cell death by a similar mechanism because its ability to kill cells was suppressed by the PARP inhibitor 3AB. This result raises the possibility that the TANK2 PARP domain, like the TANK1 PARP domain (12),

is enzymatically active, and, further, that the enzymatic activity is responsible for killing cells. This is speculation, however, since the evidence that TANK2 has PARP activity is thus far only indirect.

The ability to cause rapid cell death was the most striking difference between TANK1 and TANK2, and may underlie significant differences in their regulation or function. If the lethality is due to PARP activity, the TANK1 activity might be suppressed by relatively abundant or active cellular proteins, whereas TANK2 may be regulated by different, less abundant or less active proteins. Alternatively, compared to TANK1, TANK2 may be a more active or more easily activated PARP. It is also possible that TANK2 PARP activity is less readily inhibited by post-translational modification, such as auto-ribosylation. Similar to classical PARPs (14), TANK1 is subject to autoribosylation (12). It is not yet known whether TANK2 can autoribosylate. Finally, TANK2 may bind or ADP-ribosylate protein substrates distinct from those targeted by TANK1, and modification of TANK2 but not TANK1 substrates may cause cell death. Whatever the case, cells did not tolerate the high levels of TANK2 expression obtained in transient transfection assays, although low levels of expression obtained in stably transfected clones (not shown) were tolerated.

PARP1 is the most abundant PARP in cells, and more is known about its regulation and function than other PARPs (14). Its substrates include the components or regulators of DNA replication and repair. In addition, it binds DNA sequences that anchor chromatin to the nuclear matrix (17). PARP1 is among the first proteins to be cleaved when cells undergo apoptosis (40,41), presumably in order to conserve ATP, which is required for apoptosis. Despite a wealth of information about PARP1 substrates and the consequences of germline inactivation, there are

still major gaps in our understanding of how PARP1 functions. This is even more true for the non-classic PARPs. Substrates have been identified only for TANK1, and these are limited to TRF1 and TANK1 itself (12). Interestingly, TANK2 was recently identified as an autoantigen in several cancer patients (37,48), although the significance of this finding is not yet clear. Because both TANKs localize to the nuclear periphery, they may participate in DNA or telomere damage signaling, like the classic PARPs. On the other hand, their association with Golgi and other cytoplasmic vesicles, raises the possibility of additional non-nuclear functions.

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FIGURE LEGENDS

Figure 1. TANK2. A) Schematic representation of the human TANK1 and TANK2 proteins indicating the unique N-termini (HPS or histidine-proline-serine domain in TANK1, and NTD or N-terminal domain in TANK2) and the conserved ankyrin repeat, sterile alpha motif (SAM), and poly(ADP-ribose) polymerase (PARP) domains. B) Nucleotide and amino acid sequences of the 5' ends of the mouse and human *TNKS2* genes, revealing 96% amino acid identity, and >95% nucleotide identity in the coding region, but <83% nucleotide identity in the 5' UTR.

Figure 2. Genomic characterization. A) Radiation hybrid map localizing TANK 2 to the STS marker 10S536 on human chromosome 10. B) Genomic organization of *TNKS2*, determined from analyzing and sequencing chromosome 10 BAC clones, showing the exon-intron boundaries and the translational start and poly-adenylation sites. The approximate size of the 5' UTR is **XX** bp. The size of the 3' UTR is 2369 bp, determined by **XX** bp present in the cDNA clones and an additional **XX** bp present in several ESTs in the GenBank database. The position of the cDNA end present in the lambda clones is indicated.

Figure 3. Expression of *TNKS2* mRNA. A). Poly-A+ RNA (2 μg) from the indicated human tissues was analyzed by northern blotting for *TNKS2* mRNA, using a 1 kb cDNA probe encompassing the ankyrin domain, and β-actin mRNA (*ACTB*) to control for mRNA quantity and integrity. The β-actin probe also detects heart and muscle-specific actin mRNAs, evident as faster migrating cross-hybridizing species. B) Poly-A+ RNA (2 μg) from HT1080 human fibrosarcoma cells was analyzed by northern blotting for *TNKS2* mRNA using cDNA probes corresponding to the unique N-terminal domains of TANK1 and TANK2, shown as black bars on the diagrams below the blot.

Figure 4. *In vitro* translated and endogenous TANK2. A) Coupled in vitro transcription and translation reactions were carried out in the presence of ^{35}S-methionine using the TNKS, TNKS2 or hTERT (human telomerase catalytic component; positive control) cDNAs, or no cDNA (negative control), as indicated. The products were analyzed by SDS-PAGE, as described in Experimental Procedures. B) Protein extracts from proliferating WI-38, HT1080, and VA13 cells (60 μg) (lanes 1-3), or HT1080 transiently transfected with either a *TNKS2* (lane 4) or *TNKS* (lane 5) expression vector (30 μg) were analyzed by western blotting using affinity purified Ab-591 (lanes 1-2) or a commercial antibody (lanes 4-5). The *TNKS2* and *TNKS* expression vector backbones were pcDNA3.1 and pBK-CMV, respectively. Transient transfection efficiencies into HT1080 cells were generally >30%. Cells were lysed 6-8 h after transfection.

Figure 5. Interaction with TRF1. A) Radiolabeled HA-TRF1, TANK1 and TANK2 were produced by coupled *in vitro* transcription and translation, as described in the legend to Fig. 4

and Experimental Procedures. B) The indicated radiolabeled *in vitro* translation products (Input) were mixed together and immunoprecipitated by the indicated antibodies (IP). Lanes 1 and 3, HA-TRF1 plus untagged TANK1 or TANK2, respectively, immunoprecipitated with anti-HA. Lane 2, positive control of C-terminal V5-tagged TANK2, immunoprecipitated with anti-V5. Lane 4, HA-TRF1 plus N-terminal FLAG-tagged TANK2, immunoprecipitated with anti-HA. Lanes 5, in vitro translation reaction with no cDNA, immunoprecipitated with anti-HA. Lane 6, HA-TRF1 plus untagged TANK2, immunoprecipitated with non-specific IgG. C) TRF1 domain that interacts with TANK2. Intact TRF1 and TRF1 fragments are depicted, with the TANK1 and homodimerization domains indicated. The TRF1 cDNAs (full-length and fragments containing the indicated amino acids) were cloned into the Gal4 DNA binding domain vector pGBT9 (30). The vectors were transformed into yeast expressing pGAD-TANK2-1.7 or pGAD-TRF1 (30) (TRF1 cloned in the Gal4 activation domain vector), and interaction was assessed by a luminescent β-gal assay, as described in Experimental Procedures. For each pGBT9 construct, control luminescence (interaction with the insertless pGAD vector; Control) was 0.1-2 luminescent units and given an arbitrary value of 1.

Figure 6. Immunolocalization of epitope-tagged TANK 2. Normal human fibroblasts (A; strain 82-6) and human fibrosarcoma cells that overexpress TRF1 (30) (B; HT1080) were transiently transfected with the pcDNA3.1 expression vector containing V5-tagged TANK2, and immunostained 6 h later with anti-V5 antibody and fluorescein-conjugated secondary antibody. A representative of each cell type is shown. HT1080 cells that do not overexpress TRF1 showed an identical staining pattern (not shown). C) A representative HT1080 cell, transiently transfected with the V5-TANK2 expression vector and undergoing mitosis. The cells were

stained with anti-V5 and Texas Red-conjugated secondary antibody. Cells were counterstained with DAPI to identify the nuclei (blue fluorescence).

Figure 7. Analysis of cell death by transient transfection of TANK 2. A) Cells were incubated with the MitoCapture reagent to detect intact or collapsed mitochondrial membrane potentials. The black and white photograph shows examples of cells scored for intact (left panel; punctate red fluorescence) or permeabilized (right panel; diffuse green fluorescence) mitochondria. B) WI-38 and HT1080 cells were transiently transfected with a control (pcDNA3.1) vector or the TANK1 or TANK2 expression vectors. After 7 h, cells were stained and scored for punctate red or diffuse green fluorescence, indicative of intact or collapsed mitochondrial membrane potential respectively. Parallel cultures were transfected with a β-galactosidase expression vector, and stained for β-galactosidase activity to estimate the transfection efficiency. The percentage of cells with collapsed mitochondrial membrane potential (diffuse green fluorescence) (% Cell Death) was first normalized for transfection efficiency, and then the background (percentage of cells with green fluorescence owing to transfection of the control vector) was subtracted. The background ranged from <5% for WI-38 cells (which was given an arbitrary value of 1) to 10-15% for HT1080 cells. In the case of WI-38 cells, cells with green fluorescence were slightly less abundant after transfection of the TANK1 expression vector, compared to the control vector, giving a small negative value. C) HT1080 cells were transfected with the TANK2 expression vector, and lysates were prepared at the indicated intervals thereafter. The lysates (60 μg) were then analyzed by western blotting for PARP cleavage. Negative (-) and positive (+) controls lysates (HL-60 cells before and after etoposide treatment) were provided by the antibody

supplier (Zymed). Western blots were re-probed with an anti-actin antibody to control for protein concentration and integrity.

Figure 8. TANK2 lethality is ameliorated by 3-aminobenzamide. A) HT1080 cells were transfected with the TANK2 expression vector in the presence of increasing concentrations of 3-aminobenzimide (3AB), stained with the MitoCapture reagent 20 h later, and scored for the percentage of cells with diffuse green fluorescence (% Cell Death). The results were not normalized for transfection efficiency, which was 30-40%. B) Photomicrographs of HT1080 cells transfected with control (pcDNA3.1), TANK1 or TANK2 expression vectors, selected for 7 days with 450 μg/ml G-418 in the presence of 10 mM 3AB, followed by 3 days in medium lacking G-418 but containing 3AB.